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# Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate oxidizing bacteria

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## Abstract

Substrates that contain high ammonia levels can cause inhibition on anaerobic digestion process and unstable biogas production. The aim of the current study was to assess the effects of different ammonia levels on pure strains of (syntrophic acetate oxidizing) SAO bacteria and hydrogenotrophic methanogens. Two pure strains of hydrogenotrophic methanogens (i.e: *Methanoculleus bourgensis* and *Methanoculleus thermophiles*) and two pure strains of SAO bacteria (i.e: *Tepidanaerobacter acetatoxydans* and *Thermacetogenium phaeum*) were inoculated under four different ammonia (0.26, 3, 5 and 7g NH<sub>4</sub><sup>+</sup>-N/L) and free ammonia levels (Mesophilic: 3.31, 38.2, 63.68 and 89.15 g NH<sub>3</sub>-N/L. Thermophilic: 8.48, 97.82, 163.03 and 228.24 g NH<sub>3</sub>-N/L). The results indicated that both *T. acetatoxydans* and *T. phaeum* were more sensitive to high ammonia levels compared to the hydrogenotrophic methanogens tested. Additionally, the total incubation periods of hydrogenotrophic methanogens were significantly shorter compared to the SAO bacteria incubation periods. Thus, it seems that hydrogenotrophic methanogens could be equally, if not more, tolerant to high ammonia levels compared to SAO bacteria.

## Keywords

Ammonia inhibition; Anaerobic digestion; Biogas; Hydrogenotrophic methanogens; SAO bacteria.

## INTRODUCTION

Substrates that contain high ammonia levels can cause inhibition on the anaerobic digestion process and suboptimal biogas production (Fotidis, et al., 2014). Aceticlastic methanogenic pathway has been proven to be more sensitive to ammonia toxicity effect compared to hydrogenotrophic methanogenic coupled by syntrophic acetate oxidation (SAO) pathway (Fotidis, et al., 2013). Specifically, there are two steps in SAO pathway. First, SAO bacteria convert acetate into hydrogen and carbon dioxide. Second, hydrogenotrophic methanogens use hydrogen and carbon dioxide of the first step to produce methane (Fotidis, et al., 2013). However, there are still controversies in the literatures on ammonia tolerances of hydrogenotrophic methanogens and SAO bacteria (Fotidis, et al., 2013). Specifically, pure strains of SAO bacteria were shown to be robust to 0.6-1.0M NH<sub>4</sub>Cl (Schnürer et al., 1996; Westerholm et al., 2010). Sprott and Patel's study (1986) indicated that mesophilic hydrogenotrophic methanogens did not suffer inhibition under 0.3-0.4M ammonia. However, Fotidis et al. (2013) found that methane production of *M. bourgensis* which was cultivated in fed-batch reactors was below detection limits under 3 g NH<sub>4</sub><sup>+</sup>-N/L and hydrogenotrophic methanogens were the limiting factor for decreased growth rate of SAO co-culture. Therefore, the aim of the current study was to assess the effects of different ammonia levels on pure strains of SAO bacteria and hydrogenotrophic methanogens.

## MATERIAL AND METHODS

### Inocula

Two hydrogenotrophic methanogens (*Methanoculleus bourgensis* MS2: mesophilic *Methanoculleus thermophilus* UCLA: thermophilic) and two SAO bacteria (*Tepidanaerobacter acetatoxydans*: mesophilic, *Thermacetogenium phaeum* strain PB: thermophilic) were used as inocula in the experiments. All the pure SAO bacteria and hydrogenotrophic methanogenic strains used in this study

were obtained from DSMZ Company, Germany. They were grown in the corresponding media suggested in the literature (Westerholm, et al., 2011; Satoshi, et al., 2000; Jacob, et al., 1997) before used as inocula.

### **Experimental setup**

All hydrogenotrophic methanogens and SAO bacteria were cultivated under four different ammonia (0.26, 3, 5 and 7 g NH<sub>4</sub><sup>+</sup>-N/L) and free ammonia concentrations (Mesophilic: 3.31, 38.2, 63.68 and 89.15 g NH<sub>3</sub>-N/L. Thermophilic: 8.48, 97.82, 163.03 and 228.24 g NH<sub>3</sub>-N/L) with NH<sub>4</sub>Cl as ammonia source. To ensure identical experimental conditions, basal anaerobic media (BA) (Angelidaki et al., 1990) was used for all batch experiments. Glass batch bottles with 118 mL total and 40 mL working volume, respectively were used for hydrogenotrophic methanogens cultivation. For SAO bacteria glass test tubes with 30 mL total volume and 20 mL working volume respectively were used. 62 mL H<sub>2</sub> and 16 mL CO<sub>2</sub> were introduced into batch bottles as substrate for the hydrogenotrophic methanogens. Finally, 2 g/L acetate was used as carbon source in the SAO bacteria experiments. All the batch bottles and tubes were incubated in their corresponding temperatures (37°C for mesophilic and 55°C for thermophilic), and all experiments were performed in triplicates (n=3).

### **Analyses and calculations**

Methane quantity of hydrogenotrophic methanogens were measured with Shimadzu-14A gas chromatographer (GC). For presenting the growth of SAO bacteria, Spectronic 20D+ Spectrophotometer was used for measuring the optical density at 600 nm (OD<sub>600</sub>) (Thermoscientific, Soeborg, Denmark). The free ammonia concentrations were calculated as been described before (Fotidis, et al., 2013).

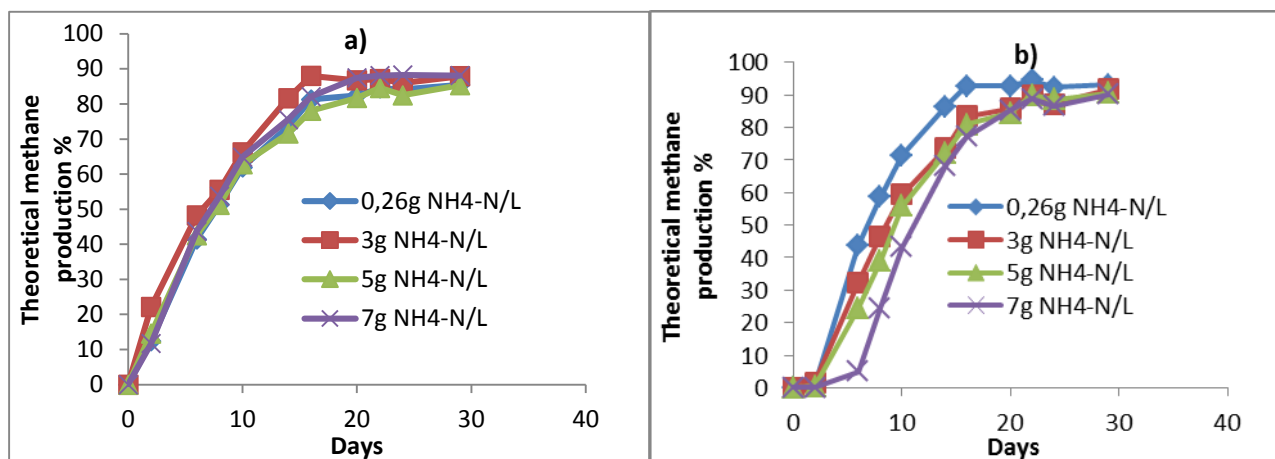
### **Analyses and calculations**

Methane content in CSTR reactors were measured with GC-TCD (MGC 82-12, Mikrolab a/s, Denmark). Volatile fatty acids (VFA) were determined using gas-chromatograph (HP 5890 series II) as described previously (Fotidis, et al., 2014). The pH was measured with PHM99 LAB pH meter. The maximum growth rate ( $\mu_{\max}$ ) of the MC culture was calculated as has been described before (Fotidis, et al., 2013a). The optical density at 600 nm (OD<sub>600</sub>) was determined with a Spectronic 20D+ Spectrophotometer (Thermoscientific, Soeborg, Denmark). All the analyses were made in triplicate (n=3) and the averages with the standard deviations ( $\pm$  SD) are presented.

## **RESULTS AND DISCUSSION**

### **Hydrogenotrophic methanogens**

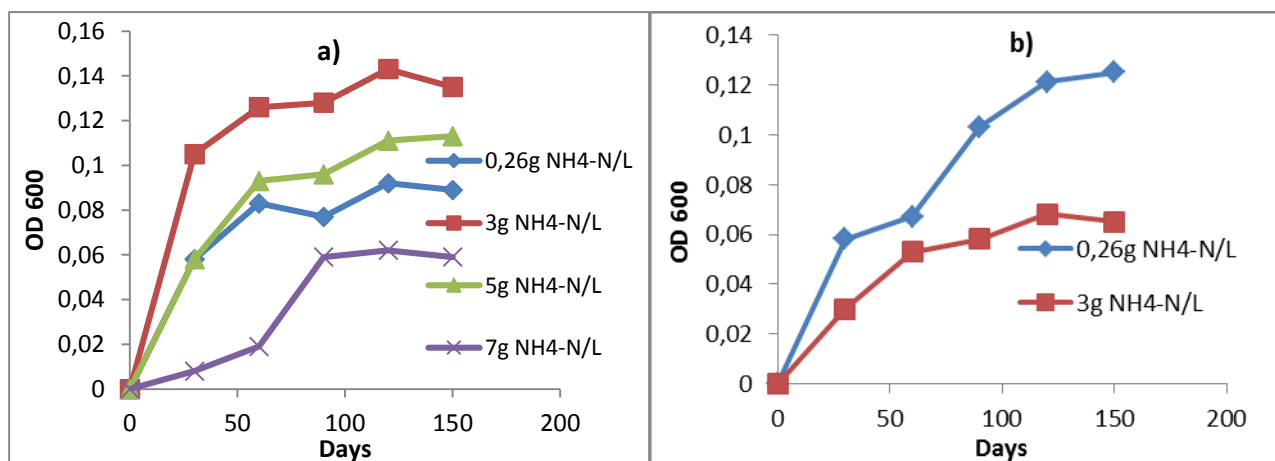
The methane production for *M. bourgensis* was not significantly ( $p>0.05$ ) affected by the increased ammonia levels (Figure 1a). The ability of *M. bourgensis* to produce methane under high ammonia concentrations has been reported before (Fotidis, et al., 2013). On contrary, methane production of *M. thermophilus* was slightly but significantly ( $p<0.05$ ) decreased, while the ammonia levels were increased (Figure 1b). This small inhibitory effect could be attributed to the higher free ammonia concentrations that the thermophilic hydrogenotrophic methanogens were exposed compared to mesophilic (Table 1) (Fotidis, et al., 2013).



**Figure 1.** The accumulative production of a) *M. bourgensis* and b) *M. thermophiles*

### SAO bacteria

The growth of SAO bacteria suffered a significant ( $p < 0.05$ ) inhibition as the ammonia concentrations were increased (Figure 2). Nevertheless, *T. acetatoxydans* was able to grow even at 7 g NH<sub>4</sub><sup>+</sup>-N/L although at much reduced rate. On contrary, the OD<sub>600</sub> of *T. phaeum* was below detection limits at 5 and 7 g NH<sub>4</sub><sup>+</sup>-N/L (Figure 2b) indicating a complete inhibition most probably caused by the high free ammonia levels (Table 1). However, these results are contradictory to a previous study (Sun et al., 2014) reported that *T. acetatoxydans* was robust to relatively high concentration (above 5 g NH<sub>4</sub><sup>+</sup>-N/L) of ammonia.



**Figure 1.** The OD<sub>600</sub> of a) *T. acetatoxydans* and b) *T. phaeum*

### Hydrogenotrophic methanogens versus SAO bacteria

The results of current study indicated that both *T. acetatoxydans* and *T. phaeum* were more sensitive to high ammonia levels compared to the hydrogenotrophic methanogens tested. Additionally, the total incubation periods of hydrogenotrophic methanogens were significantly shorter (about 15 days) compared to the SAO bacteria incubation periods (more than 120 days). This is in contrast to De Vrieze et al. (2012) who reported that hydrogenotrophic methanogens are more sensitive to ammonia inhibition compared to SAO bacteria. Thus, it seems that there are some hydrogenotrophic methanogens equally or more resistant to high ammonia levels compare to SAO bacteria.

### CONCLUSIONS

The results of the current study indicated that hydrogenotrophic methanogens could be equally, if not more, tolerant to high ammonia concentrations compared to SAO bacteria. Thus, it seems that hydrogenotrophic methanogens are the key players in the biomethanation process under high

ammonia concentrations.

## ACKNOWLEDGEMENTS

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